THE SUSCEPTIBILITY OF CACTOBLASTIS CACTORUM (BERG) TO BACILLUS THURINGIENSIS VAR. THURINGIENSIS[1,2]

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Cactoblastis cactorum (Berg), an important member of a group of gregarious tunnelling caterpillars of the family Phycitidae, has been successfully utilized for the biological control of prickly pear in Queensland, Australia (Dodd, 1940, 1959). It was introduced on Hawaii where cactus was considered a pest (Fullaway, 1954). Although no releases were made on the other Hawaiian islands, the insect rapidly spread from Hawaii to all the others (Beardsley, 1955; Bianchi, 1955, 1956; Fullaway, 1955, 1958).

C. cactorum in combination with the cochineal, *Dactylopius opuntiae* (Cockerell), has reduced stands of cactus successfully on Hawaii (Davis, 1963). On the other islands however, destruction of cactus by *C. cactorum* was not desirable since cactus was used as cattle forage during periods of drought. The insect, therefore, plays a dual role, as a beneficial species and as a pest in Hawaii. Information on its susceptibility to insect pathogens can be used both to protect or to control this insect.

Information is scarce on the effect of diseases to this insect. Ripley (1937) and Fantham (1939) found two species of protozoans infecting *C. cactorum* in South Africa. In Hawaii, a fungal pathogen (Nakao, 1953) and a cytoplasmic polyhedrosis virus (Tamashiro & Huang, 1963) were found in *C. cactorum* field populations. No detailed study has been made of the effect of any pathogen on this insect. The gregarious feeding habit of *C. cactorum* makes it a candidate for microbial control.

The present study is concerned with the pathogenicity of the bacterium, *Bacillus thuringiensis* var. *thuringiensis* to *C. cactorum*. The bacillus was found highly pathogenic to many insects, including over 140 species of Lepidoptera, some Coleoptera, Hymenoptera and Diptera (Hall, 1963; Heimple, 1960, 1963; Steinhaus, 1957; Tanada, 1959). This bacillus is commercially produced by several companies and available for field use.

MATERIAL AND METHODS

The original stock suspension of spores of *B. thuringiensis* var. *thuringiensis* was obtained from a laboratory culture of a commercial formulation. A small amount of this formulation was suspended in sterile distilled water, streaked onto nutrient agar, and held at room temperature (29 °C). After 6 days, when the bacilli had sporulated completely, the resulting colonies

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were removed and blended under sterile conditions in a Waring Blendor® with distilled water. This was necessary to break up the clumps of spores that had formed and to produce a homogeneous suspension. Concentration of spores in the stock suspension, made from this blended culture, was determined by visual counts in a Petroff-Hausser bacteria counter. It was refrigerated and dilutions were utilized for all pathogenicity tests.

Larvae in the 4th and 6th instars, molting during the previous 24 to 48 hours, were treated by the *per os* method in which a measured amount of suspension is introduced carefully into the buccal cavity of the insect with a microsyringe (Martignoni, 1955, 1959). Extreme care was necessary to avoid piercing the walls of the alimentary tract.

The larvae were removed from the cactus pads and starved for 1 or 2 hours prior to treatment so that they would not regurgitate the spore suspension. For treatment, they were anesthetized with CO_2 and microfed a fixed volume of 2μ l. for the 4th instar larvae and 4μ l. for the 6th instar larvae. Concentrations were varied to obtain the desired dosage. Control larvae were microfed with an equal volume of sterile distilled water. Ten larvae per dosage level were used for each of 3–4 replications.

In both bacteria-treated and control groups, two similarly treated larvae were placed together in a sterile ice cream carton and covered with a petri dish. Small blocks of the cactus, *Opuntia megacantha* Salm-Dyck, were included as food.

Mortality observations were made at 24 and 48 hours after treatment. Smears made from the blood and gut contents of dead larvae were examined to ascertain cause of death. Dosage mortality curves were calculated by probit analysis (Finney, 1952).

The larvae were fixed in Bouin or Carnoy's, for histopathological studies, at various times after treatment and prepared for microsection by modified methyl benzoate (Martignoni, 1957) or n-butyl alcohol (Smith, 1943) embedding techniques. Longitudinal sections were cut at 5 or 6μ and stained with Delafield's alum hematoxylin or Heindenhein's iron hematoxylin and eosin.

All of these studies were conducted at an average temperature of 26.8 °C with a range of 21.1 to 32.8 °C. The relative humidity averaged 56.3% with a range of 32 to 80%.

RESULTS AND DISCUSSION

The average larval weights of 4th and 6th instar *C. cactorum* were 26.4 mg and 107.8 mg per individual, respectively. Table 1 summarizes the pathogenicity of *B. thuringiensis* var. *thuringiensis* for *C. cactorum*. The LD-50 (median lethal dosage) for the 4th instar larvae was 6,830 spores per mg body weight with a 95% confidence interval of 5,321 to 8,766 spores per mg body weight. The formula for the dosage-mortality line was $Y = -2.277 + 1.898X$.

Table 1. The Pathogenicity of *Bacillus thuringiensis* var. *thuringiensis* to 4th and 6th Instar Larvae of *Cactoblastis cactorum*

4th Instar 26.4 mg/individual		
No. of spores per mg body wt.	No. of larvae injected	Per Cent Mortality
19318	40	87.5
9659	40	57.5
4848	40	30.0
2424	40	22.5
1212	40	10.0
0	40	0.0

6th Instar 107.8 mg/individual		
No. of spores per mg body wt.	No. of larvae injected	Per Cent Mortality
9462	30	76.7
4731	30	66.7
2365	30	43.3
1187	30	26.7
594	30	13.7
297	30	6.7
0	30	0.0

The LD-50 for the 6th instar larvae was 2,878 spores per mg body weight with a 95% confidence interval of 2,084 to 3,975 spores per mg body weight. The dosage mortality curve was $Y = -.340 + 1.544X$.

Comparison of the LD-50's of the two instars reveals an interesting phenomenon. When compared on the basis of the number of spores required per individual to attain this level of mortality (313×10^3 vs. 176×10^3), the 6th instar larvae were more resistant since more spores were required to infect these individuals.

However, when compared in terms of unit body weight, the 4th instar larvae were significantly more resistant to *B. thuringiensis* var. *thuringiensis* at $P = .01$. Age, of course, plays an important role in the determination of host susceptibility to a given pathogen but with most insects older larvae usually are more resistant to infection than young larvae. *C. cactorum* exhibited the reverse condition.

C. cactorum, however, was not as susceptible to *B. thuringiensis* var. *thuringiensis* as *Pieris rapae* (Linneaus). Tanada (1953) estimated the LD-50 for 5th instar *P. rapae* to be approximately 450 spores per mg of body weight or approximately 30×10^3 per larva. Even the more susceptible 6th instar larva of *C. cactorum* required approximately 7 times the dosage for the LD-50 and the more resistant 4th instar required 15 times the dosage.

Symptomatology

Generally, the symptoms elicited by the hosts were similar to those

exhibited by susceptible lepidopterous species (Steinhaus, 1951). The symptoms varied in degree according to the concentration of the inoculum. Larvae microfed a high concentration of spores stopped feeding within a short period, while those microfed an amount approximating the median lethal dosage continued eating up to 11 or 12 hours after treatment. Death occurred between 24 to 48 hours. Those that did not die within this period pupated.

Course of Infection and Histopathology

The course of infection by *B. thuringiensis* var. *thuringiensis* and the histopathological changes in *C. cactorum* were followed in 5th or 6th instar larvae. Both smears and microsections were made of treated hosts at 1, 2, 3, 4, 6, 12, 24, and 48 hours after treatment. Control larvae treated with sterile distilled water were smeared also and sectioned at the same times.

Smears of the fore and midgut of healthy larvae appeared almost sterile when examined microscopically, and culture plates streaked with the contents of the fore and midguts rarely showed the presence of microorganisms. Fig. 1A depicts a section of the normal midgut.

In the treated insects, however, a few short rods of the bacillus were found in the midgut within 3 to 4 hours after treatment. Also, at this time, the first visible change in the midgut epithelium, a spongy evacuolation of the cells, could be detected (Fig. 1B). As the infection progressed, the epithelial cells became detached from the basement membrane and cellular integrity was lost. The bacillus continued to multiply and 12 hours after treatment, typical *B. thuringiensis* var. *thuringiensis* chains could be found in all parts of the gut (Fig. 1C). Invasion of the hemocoel and initiation of the fatal septicemia occurred after the bacilli had increased greatly in numbers and the midgut wall showed great gaps due to the exfoliation of the epithelium (Fig. 1D).

Once in the hemocoel, the bacilli multiplied rapidly and soon caused the death of the host. Also, during this period, small short rods of a secondarily invading bacteria could be found increasing rapidly in the body cavity. The external symptom of "flaccidity" appeared during this period of intense septicemia.

In larvae dissected 48 hours after treatment, the gut was found completely destroyed, and secondary invaders were more abundant than *B. thuringiensis* var. *thuringiensis*.

The Mode of Action

Bacillus thuringiensis var. *thuringiensis* was found to produce 5 toxic substances (Krieg, 1961). Of these, the parasporal crystal, produced during sporulation, was found important to the pathogenicity of this bacillus. The crystal, soluble in alkali, causes a breakdown of the midgut epithelium of susceptible insects enabling the bacillus to invade and infect the host. The susceptible lepidopterous larvae were divided into three types on the

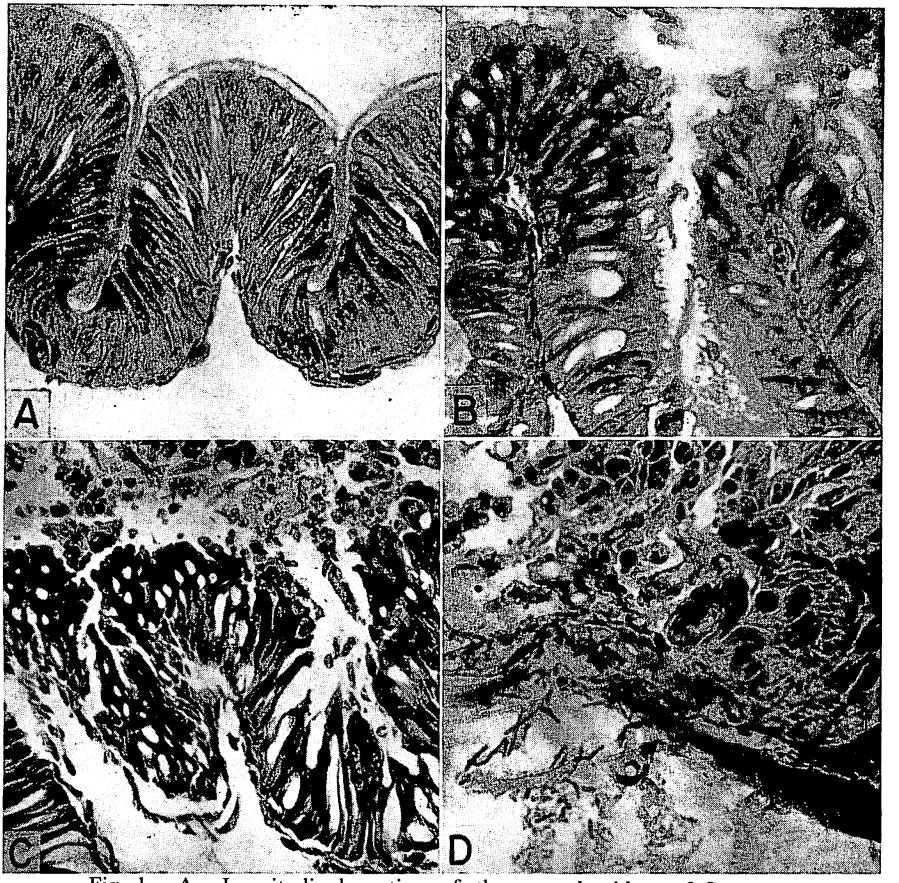

Fig. 1: A. Longitudinal section of the normal midgut of *C. cactorum*. B. Section through the same area of the midgut 3 to 4 hours after larva microfed *B. thuringiensis* var. *thuringiensis*. Note evacuolation of midgut cells. C. Same area a few hours later. Cellular integrity is lost and cells breaking away from basement membrane. D. Later stage in infection. *B. thuringiensis* var. *thuringiensis* rods can be seen penetrating into the hemocoel.

basis of their reaction to the crystal (Heimpel & Angus, 1959).

Type I insects, such as the silkworm *Bombyx mori* (Linnaeus), typically show a general and flaccid paralysis within one hour of treatment. These insects characteristically have high alkaline (pH 9–11) gut juices. The ingested crystal quickly dissolves and rapidly destroys the integrity of the mid-gut epithilium allowing the highly buffered alkaline gut contents to leak into the slightly acid blood. This results in a rapid rise in the blood pH causing a general paralysis of the host.

In the Type II insects, such as the imported cabbage worm, *P. rapae*, only the gut is paralyzed by the action of the crystals which occurs within 5 to 20 minutes after treatment. Although the gut pH in this group is also

high, there is no rapid rise in the blood pH and no general paralysis. There is, however, a slow decrease in the gut pH. Both Type I and II insects can be killed by feeding on the crystal toxin alone.

Type III insects, however, were not killed by the action of the toxin alone. Both spores and crystals were fed simultaneously to infect and kill this group. The treated insects died in 2 to 4 days without any symptoms of gut or general paralysis. The gut pH in this group did not exceed 8.9.

According to this classification system, *C. cactorum* is a Type II insect. When microfed a lethal dosage of spores and crystals, the larvae soon stopped feeding. Dissection of the treated insects revealed that the gut was paralyzed and there was no peristaltic action in the gut within a couple of hours after treatment. The insects also could not defecate. Similar results were obtained by microfeeding an extract of the crystal toxin alone. There was, however, no general paralysis in treated insects and no change in the blood pH (6.5) until after septicemia occurred.

On the other hand, there was a rapid change in the pH of the gut of the infected larva. Within 3 to 4 hours after treatment, the pH of the midgut dropped from a normal 9.3 to 8.4. The unusually rapid reduction of gut pH probably was caused by infusion of more acid blood into the gut. As the infection progressed, the pH of the gut continued to drop until at 24 hours after treatment, when septicemia occurred, the pH of the gut was down to 7.6.

Although a somewhat similar drop in gut pH could be shown in starved larvae, it was not as rapid as in treated insects. In starved larvae, there was a gradual drop in gut pH for the first 12 hours stabilizing at 8.0 and a considerable reduction in the amount of gut fluid.

Although the drop in gut pH after infection was more rapid in *C. cactorum* than in the typical Type II insect, all other evidence indicated that *C. cactorum* should be classed with that group according to its reaction to the crystal.

The Mode of Transmission

Insect pathogens vary in their routes of entry into their hosts. With bacterial pathogens, the oral route has been found to be not only the usual mode of entry, but also the most practical way of transmitting the pathogen from host to host.

C. cactorum larvae could acquire the pathogen at the time of entry into the cactus pad from a direct spray applied to the cactus or from other infected insects in the same colony. There are factors, however, that militate against pathogens applied to the surface of cactus to control *C. cactorum.* Although eggs are deposited on the outside and the larvae bore into the pad to feed, each larva does not make its individual entry hole. Instead, only a few holes, through which other larvae enter, are made. Thus many of the larvae probably would not ingest a sufficient amount of the pathogens to become infected. However, by their gregarious feeding habits, a few in-

fected larvae could contaminate the food of the healthy larvae by vomiting, or by defecating or by dying and disintegrating in the pad. If transmission could be affected from host to host by any or all of these methods, epizootics could be initiated in field populations by a few infected individuals. Therefore, several laboratory tests were conducted to determine whether diseased larvae could transmit the pathogen in a virulent state to healthy larvae.

In the first test, treated larvae in various stages of disease were placed in the same pad with healthy larvae. No healthy larva became infected with *B. thuringiensis* var. *thuringiensis* although equal numbers of each were placed together, an indication that such association does not spread infection.

In the second test, ten 4th instar larvae, killed by *B. thuringiensis* var. *thuringiensis*, were ground with 10 ml of sterile distilled water and the resultant suspension was used to coat the external surfaces of cactus pads. Twenty-five 4th and an equal number of 5th instar larvae were allowed to feed on these pads. Although 2 of the 4th and 6 of the 5th instar larvae died, examinations revealed that mortality was not attributable to the bacillus. Subsequently, 4 or 8 microliters of a similar inoculum was injected directly into the buccal cavities of 20 late 5th instar larvae. Again no mortality was attributable to *B. thuringiensis* var. *thuringiensis*.

In the third test, the concentration of the inoculum was doubled and 20 bacillus-killed larvae were triturated with 10 ml of sterile distilled water. Twelve microliters of this suspension were microfed again to the hosts and although three out of ten died, deaths were not caused by the bacillus. Also, this same inoculum was smeared on cactus and injected directly into the pad where the hosts were feeding. In both of these trials, there was no mortality.

These tests showed that *B. thuringiensis* var. *thuringiensis* obtained directly from dead or dying *C. cactorum* was not pathogenic to healthy hosts even when massive amounts of highly concentrated suspensions were injected directly into the alimentary tract of the host insect. The negligible mortality in treated insects apparently was due to other "secondary bacteria" which showed some pathogenicity when microfed in such massive concentrations along with the degradation products of the dead host.

Since *B. thuringiensis* var. *thuringiensis* derived from *C. cactorum* showed such a lack of virulence, further tests were conducted to determine reasons for this phenomenon. At one day intervals after larvae were microfed a lethal dosage of *B. thuringiensis* var. *thuringiensis*, some were triturated in sterile distilled water and the resultant suspension checked for bacilli. A sample of this suspension was cultured on nutrient agar. The results revealed that in larvae triturated at one and two days after treatment, the bacillus was abundant and easily found in both smears and cultures. Three days after treatment, however, when all the treated larvae were dead, no bacillus was seen in the smears and only a few colonies appeared in the

cultures. After five days, even the nutrient agar cultures *B. thuringiensis* var. *thuringiensis* had no colonies. A second test verified these results.

The bacillus was found for slightly longer periods in suspensions made of hosts triturated immediately after death, which occurred within one or two days after treatment, and held at room temperature. Culture tests again showed no viable *B. thuringiensis* var. *thuringiensis* after nine days.

B. thuringiensis var. *thuringiensis*, therefore, was unable to survive in the cadavers of *C. cactorum* for more than five days or for more than nine days in suspensions made immediately after the host died. The reason for this inability to survive in dead hosts or suspensions was obvious after several hundred *Bacillus*-killed *C. cactorum* were observed carefully. *B. thuringiensis* var. *thuringiensis* is not able to sporulate in *C. cactorum*. In none of the dead hosts or suspensions was sporulation ever observed.

C. cactorum apparently is an abnormal host of the bacillus. There may be either some factor or factors essential to sporulation absent in *C. cactorum* or there may be inhibitory factors preventing sporulation. That the bacillus derived from dead *C. cactorum* can sporulate in an adequate medium was shown by transferring the bacillus from the dead hosts to nutrient agar. In these nutrient agar cultures, growth of the bacillus was profuse and sporulation occurred in the normal time. Moreover, the *B. thuringiensis* var. *thuringiensis* derived from these agar cultures was pathogenic to *C. cactorum*.

This inability of *B. thuringiensis* var. *thuringiensis* to sporulate in *C. cactorum* not only explains the lack of baccillus survival but also precludes the initiation of epizootics in the field by infecting a few individuals or by treating a small area and expecting the disease to spread into adjacent untreated areas. Since the bacillus cannot sporulate in *C. cactorum*, it does not produce the protein crystal. Without this crystal, *B. thuringiensis* var. *thuringiensis* is unable to invade and infect *C. cactorum*.

SUMMARY AND CONCLUSIONS

The susceptibility of *Cactoblastis cactorum*, an insect that is both a pest and a beneficial species in Hawaii, to *Bacillus thuringiensis* var. *thuringiensis* was investigated. The LD-50 for the 4th instar larva was 6,830 spores per mg body weight, or 176×10^3 spores/larva and 2,878 spores per mg body weight, or 313×10^3 spores/larva for the 6th instar. When compared on basis of weight, the 6th instars were significantly ($P=0.01$) more susceptible to the bacillus than the 4th instars. Symptoms of infected larvae were similar to those described for other infected lepidopterous larvae. Histopathological studies showed typical midgut breakdown followed by hemocoelic invasion by the bacilli. *C. cactorum* was a Type II insect according to the classification of Heimple and Angus. There was no general paralysis in the treated insects, but the gut was paralyzed shortly after treatment. The gut pH was high (9.0 to 9.3), and the blood was slightly acid (6.6 to 6.4). After treatment with *B. thuringiensis* var. *thuringiensis* there was a gradual decrease in the gut pH.

Field transmission of *B. thuringiensis* var. *thuringiensis* from larva to larva appears highly improbable. Not only does the mode of feeding enable them to escape most of the pathogens that may be on the surface of the cactus, but, more important, *B. thuringiensis* var. *thuringiensis* does not sporulate in *C. cactorum* and without the crystal toxin produced at sporulation, the bacillus is unable to invade and infect additional hosts.

LITERATURE CITED

BEARDSLEY, J. W. 1955. Notes and exhibitions. PROC. HAW. ENT. SOC. 15: 387.

BIANCHI, F. A. 1955. Notes and exhibitions. IBID. 15: 385. 1956. Notes and exhibitions. IBID. 16:16.

DODD, A. P. 1940. The biological campaign against prickly-pear. Brisbane, Queensland. 177 pp.

——1959. The biological control of prickly-pear in Australia. BIOGEOGRAPHY & ECOLOGY IN AUSTRALIA, Monographiae Biologicae. 8: 565–77.

FANTHAM, H. B. 1939. *Nosema cactoblastis*, sp. n., and *Nosema cactorum*, n. sp. microsporidian parasites of species of Cactoblastis (Lepidoptera) destructive to prickly-pear. PROC. ZOOL. SOC. LOND. (B): 689–705.

FINNEY, D. J. 1952. Probit analysis. 2nd ed. Cambridge University Press. Cambridge. 318 pp.

FULLAWAY, D. T. 1954. Biological control of cactus in Hawaii. JOUR. Econ. ENT. 47: 696–700.

——1955. Notes and exhibitions. PROC. HAW. ENT. SOC. 15: 381.

——1958. Notes and exhibitions. IBID. 16: 336.

GATENBY, J. B. AND H. W. BEAMS. 1950. The microtomist vade-mecum. 11th ed. J. & A. Churchill Ltd., London. xiv+753 pp.

HALL, I. M. 1963. Microbial control. *In* "Insect Pathology, an advanced treatise", Vol. 2, (E. A. Steinhaus, ed.) Academic Press, New York, 477–517.

HEIMPEL, A. M. AND T. A. ANGUS. 1959. The site of action of crystalliferous bacteria in Lepidoptera larvae. JOUR. INSECT PATHOL. 1: 152–70.

——1960. Bacterial insecticides. BACTERIOL. REV. 24: 266–88.

——1963. Diseases caused by certain sporeforming bacteria. *In* "Insect Pathology, an advanced treatise", Vol. 2, (E. A. Steinhaus, ed.) Academic Press, New York. pp. 457–97.

MARTIGNONI, M. E. 1955. Microinjector needle for determination of "*per os*" LD-50 of insect viruses. SCIENCE 122: 764.

——1959. Preparation of glass needles for microinjection. JOUR. INSECT. PATHOL. 1: 294–96.

NAKAO, H. 1953. Notes and exhibitions. PROC. HAW. ENT. SOC. 15: 7.

RIPLEY, L. B. 1937. *Nosema* disease of *Cactoblastis*. FARMING IN SOUTH AFRICA 12: 325, 345.

SMITH, S. G. 1943. Techniques for the study of insect chromosomes. CAN. ENTOMOLOGIST 75: 21–34.

STEINHAUS E. A. 1951. Report of diagnosis of diseased insects 1944–1950. HILGARDIA 20: 629–78.

——1957. List of insects and their susceptibility to *Bacillus thuringiensis* Berliner and other spore forming bacteria. Univ. of Calif. (Berkeley), LAB. INSECT PATHOL. MIMEO. SER. 4, 24 pp.

TAMASHIRO, M. AND S. S. HUANG. 1963. A cytoplasmic polyhedrosis of *Cactoblastis cactorum* (Berg). JOUR. INSECT PATHOL. 5: 398–99.

TANADA, Y. 1953. Susceptibility of the imported cabbage worm to *Bacillus thuringiensis* Berliner. PROC. HAW. ENT. SOC. 15: 159–66.

——1959. Microbial control of insect pests. ANN. REV. ENTOMOL. 4: 277–302.